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Urinary excretion of endothelin-1 in normal subjects and patients with renal disease

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Urinary excretion of endothelin-1 in normal subjects and patients with renal disease. To elucidate the pathophysiological significance of urinary endothelin-1 (ET-1), we measured urinary excretion of ET-1-like immunoreactivity (LI) in 17 patients with renal disease and 9 normal subjects. Twenty-four hour urinary ET-1-LI excretion in patients with renal disease (358 ± 68 ng, mean ± SE) was significantly ($P < 0.005$) greater than that of normal subjects (77 ± 5 ng). In patients with renal disease, ET-1-LI clearance (C_{ET}) exceeded creatinine clearance (C_{CR}); C_{ET}/C_{CR} (305 ± 81%) was significantly ($P < 0.005$) greater than that of normal subjects (43 ± 13%). The 24-hour urinary excretion of ET-1-LI in patients with renal disease showed significant correlation with that of N-acetyl-β-D-glucosaminidase ($r = 0.587$, $P < 0.05$), β_2-microglobulin ($r = 0.614$, $P < 0.01$) and albumin ($r = 0.484$, $P < 0.05$). Intravenous infusion of saline (500 ml) in seven normal subjects did not affect urinary ET-1 excretion rate. These data suggest that urinary excretion of ET-1 derives mainly from renal tubular secretion at least in patients with renal disease, and that degradation and/or reabsorption of ET-1 at the tubular site may also contribute to the renal handling of ET-1. Therefore, urinary excretion of ET-1 should serve as a potential marker for renal injury.

Endothelin-1 (ET-1) is a 21-residue vasoconstrictor/pressor peptide, originally isolated and sequenced from porcine aortic endothelial cells [1]. Using a specific radioimmunoassay (RIA) for ET-1, we have demonstrated the presence of ET-1-LI in human plasma [2] as well as in urine [3]. We have further shown that renal epithelial cell lines ($LLCPK_1$ and MDCK) also synthesize and secrete ET-1-LI into the medium [4]. Assuming that ET-1 and related peptides are secreted from renal tubular cells into the lumen in vivo, the physiological and pathophysiological functions of urinary ET-1 need to be elucidated. Furthermore, measurement of urinary excretion of N-acetyl-β-D-glucosaminidase (NAG), β_2-microglobulin (β_2m), or albumin proves to be useful markers to detect tubular and/or glomerular disorders [5,6]. Therefore, the present study was designed to determine the urinary excretion of ET-1-LI in patients with renal disease compared with that of normal subjects. We also compared urinary excretion of ET-1-LI with that of NAG, β_2m and albumin to know whether urinary ET-1 could be a potential marker for renal injury. Lastly, we studied whether volume expansion affects urinary excretion rate of ET-1-LI in normal subjects.

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Methods

Subjects

Nine healthy volunteers (6 men and 3 women; aged 38 ± 6 years) and 17 patients with renal disease (12 men and 5 women, aged 44 ± years) participated in the present study (Table 1) Informed consent was obtained from each individual. Renal disease consisted of chronic glomerulonephritis (membranous proliferative glomerulonephritis, IgA nephropathy, minimal change disease, focal glomerulosclerosis), lupus nephritis, renal transplantation from cadaver, and end-stage renal disease of unknown etiology, as summarized in Table 1.

Creatinine clearance (C_{Cr}) of normal control subjects and patients with renal disease were 105 ± 4 ($N = 9$) and 54 ± 10 ($N = 17$) ml/min/1.73 m^2, respectively. The urinary excretion of albumin in normal subjects and patients with renal disease were 0.005 ± 0.001 and 2.7 ± 0.8 g/24 hr, respectively.

Saline loading

Saline was infused i.v. for one hour (total volume 500 ml) in seven normal subjects (aged 32 ± 2 years). Urine specimens were collected for a two hour period before and after saline infusion. Blood sampling was performed in the middle of each urine collection period.

Sample collection

Urine was collected during a complete 24-hour period from normal subjects and patients with renal disease. Blood samples were simultaneously obtained early in the morning on the day of urinary sampling from all normal subjects and 13 patients with renal disease. Aliquots of urine and plasma were frozen at −40°C until assayed.

Radioimmunoassay (RIA) of ET-1

Urinary and plasma ET-1-LI was measured by specific radioimmunoassay (RIA) as recently described [2,3]. The antibody used cross reacted fully with ET-1, 2% with big ET-1 and less than 0.1% with ET-2 or ET-3. Urine samples were serially diluted and assayed. Plasma samples were extracted with octadecyl-silica (Spe-C_8, J.T. Baker Chemical Co., Phillipsburg, New Jersey, USA) as described in [3]. The recovery of

Table 1. Summary of clinical data in patients with renal disease compared with normal subjects studied

Case	Age/Sex	Disease	C_{Cr} *ml/min/1.73 m²*	MAP *mm Hg*	UFR *ml/min*	$U_{Na}V$ *mEq/24 hr*	$U_{ET}V$ *ng/24 hr*	C_{ET} *ml/min/1.73 m²*
1	53/M	MPGN	53	130	0.97	92	152	29
2	56/F	MPGN	128	108	1.30	85	98	32
3	65/M	MPGN	21	86	0.77	43	139	—
4	20/M	IgA nephropathy	118	89	0.94	167	164	330
5	35/M	IgA nephropathy	69	97	0.83	96	121	—
6	64/F	IgA nephropathy	26	71	1.53	160	295	46
7	53/F	MCD	115	104	0.61	62	74	74
8	21/M	MCD	108	84	1.08	31	65	91
9	29/M	MCD	76	117	1.34	163	115	—
10	62/M	FGS	15	147	0.92	89	667	31
11	37/M	lupus nephritis	44	128	0.90	60	312	32
12	46/F	lupus nephritis	71	96	1.11	176	512	302
13	17/M	end-stage RD	10	103	0.58	42	681	88
14	47/M	end-stage RD	8	114	0.90	61	377	128
15	25/M	end-stage RD	7	113	1.01	73	824	61
16	72/F	end-stage RD	5	92	0.63	63	630	—
17	35/M	transplantation	37	105	3.25	234	860	128
Mean ± SE	44 ± 4		54 ± 10[a]	105 ± 5[b]	1.63 ± 0.55	100 ± 14	358 ± 68[a]	106 ± 28[c]
Normal ($N = 9$)	38 ± 6 (years old)		105 ± 4	88 ± 4	0.92 ± 0.14	145 ± 29	77 ± 5	50 ± 18

Abbreviations are: C_{Cr}, creatinine clearance; MAP, mean arterial blood pressure; UFR, urinary flow rate; $U_{Na}V$, urinary sodium excretion; $U_{ET}V$, urinary ET-1-LI excretion; C_{ET}, ET-1-LI clearance; MPGN, membranous proliferative glomerulonephritis; MCD, minimal change disease; FGS, focal glomerulosclerosis; RD, renal disease.

Significant differences between normal subjects and patients are denoted by [a]$P < 0.005$, [b]$P < 0.025$, and [c]$P < 0.05$.

standard ET-1 during the extraction procedure was 61.8 ± 3.4% ($N = 4$). In brief, 0.1 ml standard ET-1 or sample and 0.1 ml antibody (final dilution, 1:150,000) were preincubated at 4°C for 24 hours, followed by the addition of 0.1 ml [^{125}I]ET-1 (specific activity: 2000 Ci/mmol, Amersham, Japan) and further incubated for 48 hours. Separation of the bound from free ligand was accomplished by the double antibody method. The sensitivity of the ET-1 RIA was 1 fmol/tube, and the 50% intercept was 8 fmol/tube. Intraassay and interassay coefficients of variations were 6.9% and 7.3%, respectively.

Determinations of other urinary markers

Urinary N-acetyl-β-D-glucosaminidase (NAG), β_2-microglobulin (β_2m), and albumin were determined by colorimetric analysis, Latex photometric immunoassay, and radioimmunoassay, respectively. Urinary and plasma creatinine concentrations were measured by an Autoanalyzer (Hitachi 736 model).

Statistical analysis

Values are given as the mean ± standard error (SE). Statistical analysis was performed using two sample Wilcoxon test and paired *t*-test.

Results

The serial dilution curves generated by urine samples from patients with massive proteinuria (2.9~7.9 mg/ml) and normal subjects were parallel to that of standard ET-1 in RIA (Fig. 1), suggesting that material(s) immunologically similar, if not identical, to ET-1 exists in human urine. The percentage of nonspecific binding in the absence of antibody to the total radioactivity added into the urine samples from patients with renal disease (4.6 ± 0.6%; $N = 8$) was not different ($P > 0.05$) from that of control samples (5.1 ± 0.2%). The addition of albumin (concentrations up to 10 mg/ml) into incubation mixture did not affect the binding of [^{125}I]ET-1 to its antibody in RIA (data not shown). Furthermore, there existed an excellent correlation ($r = 0.983$) between the values in extracted and unextracted urine samples [3]. Therefore, the extraction procedure is not required for the routine measurement of urinary ET-1-LI.

Patients with renal disease showed a significantly ($P < 0.005$) greater daily urinary ET-1-LI excretion (358 ± 68 ng) than that of normal subjects (77 ± 5 ng; Table 1, Fig. 2A). The clearance of ET-1-LI (C_{ET}) in normal subjects (50 ± 18 ml/min/1.73 m²) was less than C_{Cr} (105 ± 4 ml/min/1.73 m²), while C_{ET} (106 ± 28) exceeded C_{Cr} (57 ± 13) in 13 patients with renal disease (Table 1). As shown in Figure 2B, the calculated ratio of ET-1-LI to creatinine clearance (C_{ET}/C_{Cr}) in renal disease (305 ± 81%) was significantly ($P < 0.005$) greater than that of normal subjects (43 ± 13%).

The 24-hour urinary excretion of ET-1-LI in patients with renal disease showed significant correlations with that of NAG ($r = 0.587$, $P < 0.05$), β_2m ($r = 0.614$, $P < 0.01$) and albumin ($r = 0.484$, $P < 0.05$ Fig. 3), but not with mean arterial blood pressure ($r = 0.226$), urine flow rate ($r = 0.327$), or sodium excretion ($r = 0.204$, Table 1).

Intravenous saline infusion in seven normal subjects significantly ($P < 0.001$) increased urine flow rate from 0.92 ± 0.08 to 1.82 ± 0.18 ml/min as well as sodium excretion rate from 0.17 ± 0.03 to 0.29 ± 0.03 mEq/min ($P < 0.01$). Urinary ET-1-LI excretion rate before (117 ± 17 pg/min) and after saline infusion (133 ± 19 pg/min) did not change (Fig. 4). Neither plasma ET-1-LI levels (2.0 ± 0.2 vs. 1.8 ± 0.4 pg/ml) nor C_{Cr} (133 ± 6 vs. 134 ± 10 ml/min/1.73 m²) changed during saline infusion.

Discussion

The present study confirms our previous observations that ET-1-LI is excreted in normal subjects [3], and further shows that urinary excretion of ET-1-LI is greater in patients with

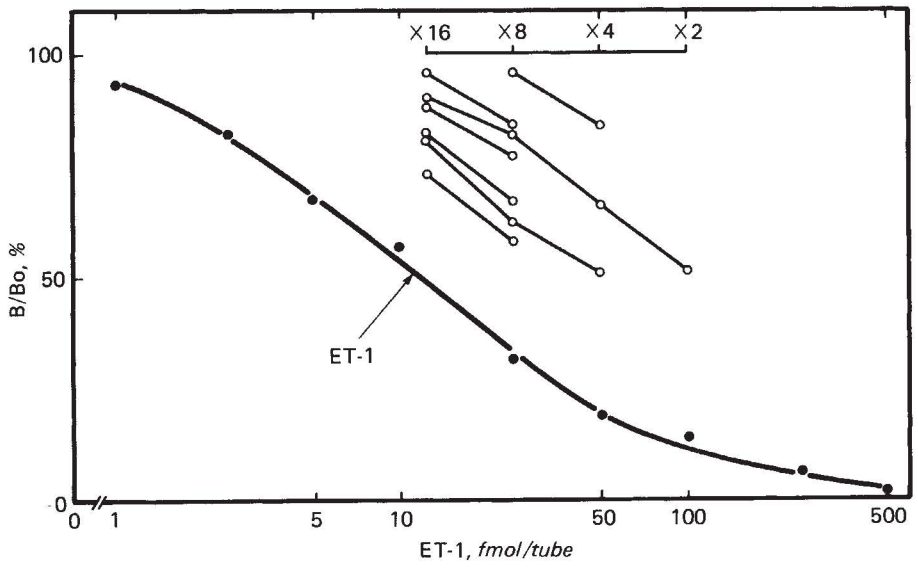

Fig. 1. *Dilution curves of human urine in ET-1 RIA.* Urine samples (○) obtained from renal disease patients with massive proteinuria were serially diluted and compared with that of standard curve of ET-1 (●).

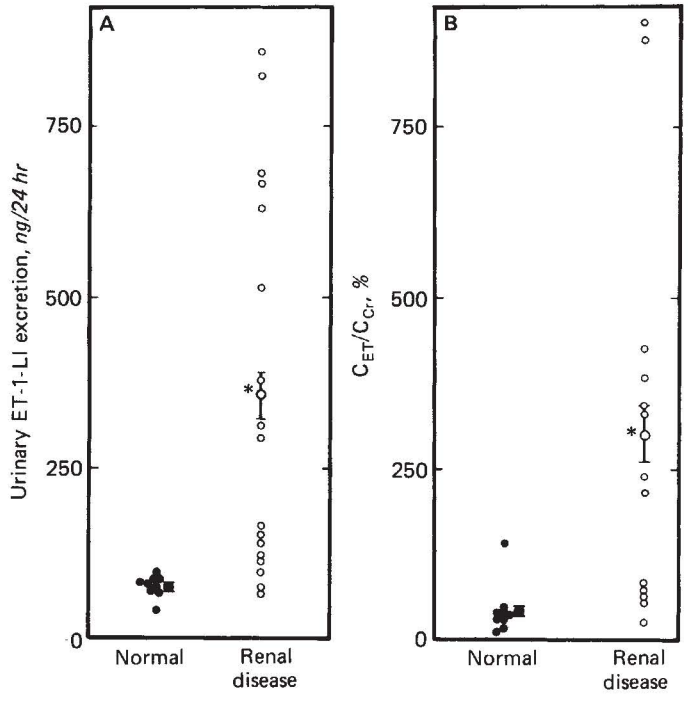

Fig. 2. *Urinary ET-1-LI excretion* (**A**) *and the ratio of ET-1-LI clearance to creatinine clearance* (**B**). Patients with renal disease (○) showed significantly ($*P < 0.005$) higher excretion of ET-1-LI and C_{ET}/C_{Cr} than those of normal subjects (●).

renal disease than in normal subjects. Results of the present study show that high concentrations of albumin did not affect the binding of [^{125}I]ET-1 to its antibody, and the fact that nonspecific binding of urine samples from patients with renal disease did not differ from that of control samples suggests that albumin and/or other substance(s) in renal disease patients' urine interfers little, if any, in ET-1 RIA. The apparent parallelism of the dilution curves of the unextracted urine samples from renal disease patients with proteinuria as well as normal subjects to that of standard ET-1 in RIA, further strengthens the conclusion that ET-1-like material(s) in human urine is immunologically indistinguishable from authentic ET-1. Furthermore, we have already shown that an excellent correlation exists between urinary ET-1-LI levels measured directly and those after extraction [3]. Therefore, it appears most unlikely that increased urinary excretion of ET-1-LI in patients with renal disease is simply due to increased excretion of nonspecific ET-1-binding material(s).

In the present study, urinary ET-1-LI excretion rate did not change after saline infusion in normal subjects, suggesting that urinary ET-1-LI excretion is not influenced by urine volume. The ratio of C_{ET}/C_{Cr} was less than 100% in all but one normal subjects. Should ET-1 be freely filtrable through the glomeruli, and all the filtered ET-1 be excreted in the final urine without any secretion from or reuptake by the tubules, the ratio of C_{ET}/C_{Cr} should be 100%. Thus, the present clearance data suggest a possible degradative and/or reabsorptive process of ET-1 at the tubular level. Our data are consistent with those of recent studies in which bilateral nephrectomy of rats leads to delayed disappearance of exogenous ET-1 [8] and potentiation of its vasoconstriction [9], suggesting the importance of kidney as the site of clearance for ET-1.

In 8 of 13 patients with renal disease, however, the ratio of C_{ET}/C_{Cr} exceeded 100%. This observation suggests that ET-1 may be secreted mainly from the renal tubular cells into lumen. In fact, we have demonstrated the secretion of ET-1-LI from renal tubular cell lines (LLCPK$_1$ and MDCK) [4], and the expression of preproET-1 mRNA in the cells [7].

The positive, although weak, correlation between urinary excretion of ET-1-LI and albumin seems to argue against its tubular source, but for its glomerular source. However, urinary excretion of NAG with a molecular size of 125,000, that is, too large to be filtered, also correlates closely with proteinuria in infants with renal disease [10]. In fact, our adult nephrotic patients excreted far higher rates of the tubular marker enzyme, NAG and protein, β_2m than those with normal renal function. Furthermore, urinary ET-1-LI excretion in our patients corre-

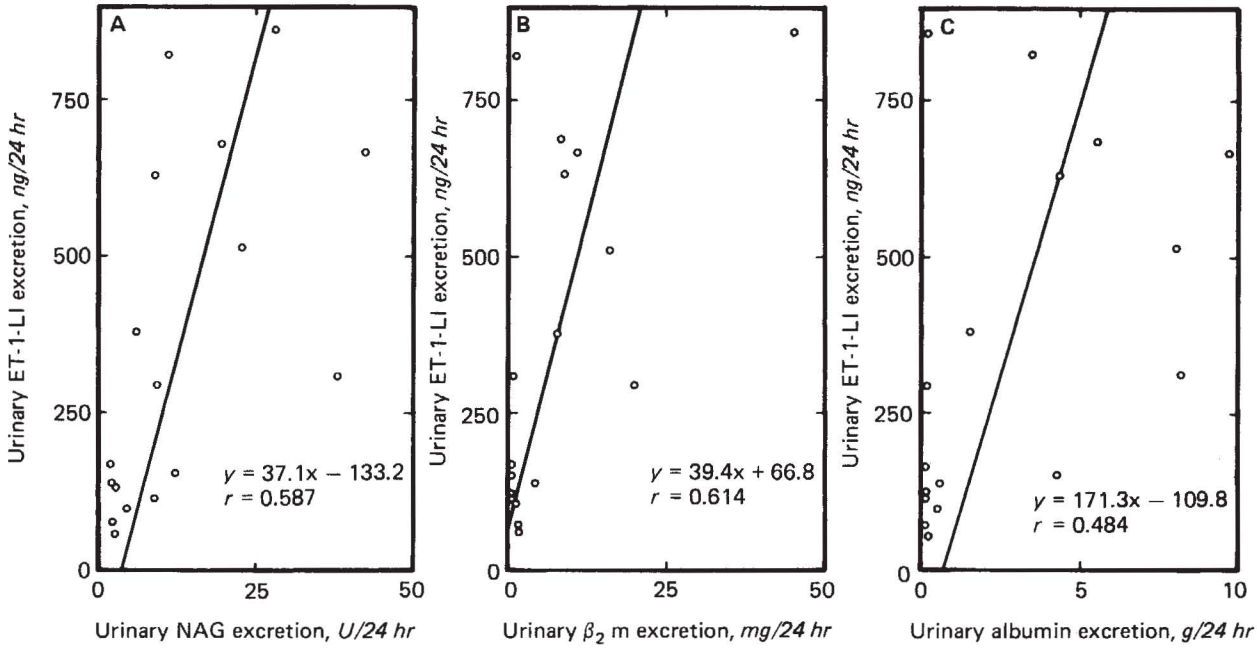

Fig. 3. *Correlations between urinary excretion of ET-1-LI,* **(A)** *N-acetyl-β-D-glucosaminidase (NAG),* **(B)** *β_2-microglobulin (β_2m) and* **(C)** *albumin in patients with renal disease.* The coefficients of correlation (r) of ET-1-LI excretion with NAG, β_2m and albumin were 0.587 ($P < 0.05$), 0.614 ($P < 0.01$), and 0.484 ($P < 0.05$), respectively.

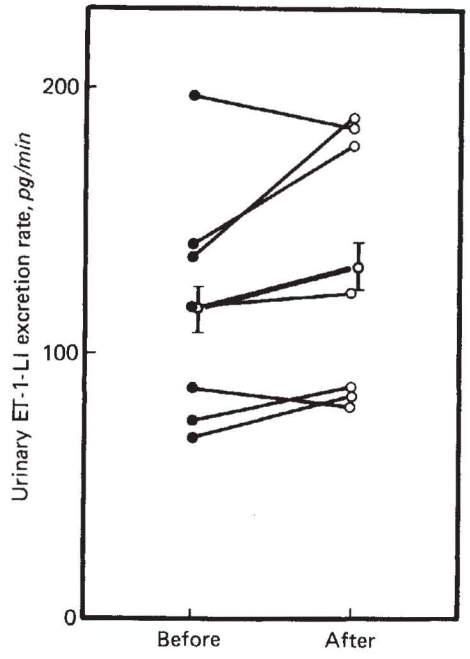

Fig. 4. *The change of urinary ET-1-LI excretion rate during saline infusion.* Uinary ET-1-LI excretion did not significantly change before and after saline (500 ml) infusion in 7 normal subjects.

lated more closely with that of NAG and β_2m than albumin. Thus, the mechanism of the increased excretion of urinary ET-1-LI similar to that of NAG and β_2m might be responsible in our nephrotic patients.

The physiological function of ET-1 in the kidney remains unknown. It has recently been shown that ET-1 inhibits Na^+-K^+-ATPase in inner medullary collecting duct [11] as well as vasopressin-induced cyclic AMP accumulation in the collecting duct [12] of rat kidney. Therefore, it is tempting to speculate that ET-1 of renal origin may function as a local modulator of ion transport/water permeability in renal tubules. Furthermore, it has been reported that ET-1 acts as a mitogenic factor for glomerular mesangial cells [13]. If ET-1 also has a mitogenic effect on tubular cells, the increased urinary ET-1-LI in renal disease might participate in the healing process of tubular cells, such as in glomerulonephritis or interstitial nephritis. It is thus significant that several active substances released during coagulation and inflammatory process, such as thrombin, platelet-derived transforming growth factor-β and cytokines (tumor necrotizing factor-α, interleukin-1β), stimulate ET-1-LI secretion from renal epithelial cells in culture [7]. Therefore, urinary ET-1 excretion, like NAG and β_2m, may serve as a potential marker for renal injury.

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